LUMINOGENIC AND NONLUMINOGENIC MULTIPLEX ASSAY

Background of the Invention

Luminescence is produced in certain organisms as a result of a luciferase-mediated oxidation reaction. Luciferase genes from a wide variety of vastly different species, particularly the luciferase genes of *Photinus pyralis* and *Photuris pennsylvanica* (fireflies of North America), *Pyrophorus plagiophthalamus* (the Jamaican click beetle), *Renilla reniformis* (the sea pansy), and several bacteria (e.g., *Xenorhabdus luminescens* and *Vibrio spp*), are extremely popular luminescence reporter genes. Firefly luciferase is also a popular reporter for determining ATP concentrations, and, in that role, is widely used to detect biomass. Luminescence is also produced by other enzymes when those enzymes are mixed with certain synthetic substrates, for instance, alkaline phosphatase and adamantyl dioxetane phosphate, or horseradish peroxidase and luminol.

Luciferase genes are widely used as genetic reporters due to the non-radioactive nature, sensitivity, and extreme linear range of luminescence assays. For instance, as few as 10^{-20} moles of firefly luciferase can be detected. Consequently, luciferase assays of gene activity are used in virtually every experimental biological system, including both prokaryotic and eukaryotic cell cultures, transgenic plants and animals, and cell-free expression systems. Similarly, luciferase assays used to determine ATP concentration are highly sensitive, enabling detection to below 10^{-16} moles.

Luciferases can generate light via the oxidation of enzyme-specific substrates, e.g., luciferins. For firefly luciferase and all other beetle luciferases, light generation occurs in the presence of magnesium ions, oxygen, and ATP. For anthozoan luciferases, including *Renilla* luciferase, only oxygen is required along with the substrate coelentrazine. Generally, in luminescence assays to determine genetic activity, reaction substrates and other luminescence activating reagents are introduced into a biological system suspected of expressing a reporter enzyme. Resultant luminescence, if any, is then measured using a luminometer or any suitable radiant energy-measuring device. The assay is very rapid and sensitive, and

provides gene expression data quickly and easily, without the need for radioactive reagents.

Luciferases are one of a number of reporters, e.g., firefly luciferase, *Renilla* luciferase, chloramphenicol acetyl transferase (CAT), beta-galactosidase (lacZ), beta-glucuronidase (GUS) and various phosphatases, such as secreted alkaline phosphatase (SEAP) and uteroferrin (Uf; an acid phosphatase), that have been combined and used as co-reporters of genetic activity. A dual enzyme reporter system relates to the use, expression, and measurement of two individual reporter enzymes within a single system. In genetic reporting, dual reporter assays are particularly useful for assays in individual cells or cell populations (such as cells dispersed in culture, segregated tissues, or whole animals) genetically manipulated to simultaneously express two different reporter genes. Most frequently, the activity of one gene reports the impact of the specific experimental conditions, while the activity of the second reporter gene provides an internal control by which all sets of experimental values can be normalized. Dual enzyme reporter technology can also be employed with cell-free reconstituted systems such as cellular lysates derived for the simultaneous translation, or coupled transcription and translation, of independent genetic materials encoding experimental and control reporter enzymes. Immunoassays may, likewise, be designed for dual reporting of both experimental and control values from within a single sample.

The performance of any dual enzyme reporter assay is based on the characteristics of the constituent enzyme chemistries and the ability to correlate their respective resulting data sets. Disparate enzyme kinetics, assay chemistries and incubation requirements of various reporter enzymes can complicate combining two reporter enzymes into an integrated, single tube or well dual reporter assay format. One approach to integration of a dual reporter assay is described in U.S. Patent No. 5,744,320, which discloses particular general or specific quenching agents for beetle and *Renilla* luciferase assays and demonstrates an exemplary dual reporter assay for sequentially determining luminescence from firefly luciferase then *Renilla* luciferase. Similarly, U.S. Patent No. 6,586,196 discloses several dual reporter assay systems. Like the dual reporter systems disclosed in the '320 patent,

luminescence is the measurable product of each of two reactions in the '196 patent. Approaches to multiplexing of reporter assays which incorporate not only different substrates but also different detection technologies are described in Liu et al. (2000) and Qazi et al. (2002). For instance, Liu et al. report luciferase and GFP activity in the same organism, where enzyme activity is determined via luminescence and fluorescence detection, respectively, in a stepwise fashion.

Reporters are also useful to detect the presence or activity of molecules within cells or supernatants. For instance, proteases constitute a large and important group of enzymes involved in diverse physiological processes such as protein turnover in blood coagulation, inflammation, reproduction, fibrinolysis, and the immune response. Numerous disease states are caused by, and can be characterized by, the alterations in the activity of specific proteases and their inhibitors. The ability to measure these proteases in research or in a clinical setting is significant to the investigation, treatment and management of disease states. For example, caspase-3 and caspase-7 are members of the cysteine aspartyl-specific protease (also known as the aspartate specific-cysteine protease, "ASCP") family and play key effector roles in cell death in mammalian cells (Thornberry et al., 1992; Nicholson et al., 1995; Tewari et al., 1995; and Fernandes-Alnemri et al., 1996).

Proteases, however, are not easy to assay with their naturally occurring substrates. Moreover, many currently available synthetic substrates are expensive, insensitive, and nonselective.

Numerous chromogenic and fluorogenic substrates have been used to measure proteases (Monsees et al., 1994; Monsees et al., 1995) and modified luciferins have provided alternatives to fluorescent indicators (U.S. Patent Nos. 5,035,999 and 5,098,828). Methods for using modified luciferins with a recognition site for a hydrolase as a pro-substrate were first described by Miska and Geiger (1989), where heterogeneous assays were conducted by incubating a modified luciferin with a hydrolase for a specified period of time, then transferring an aliquot of the mixture to a solution containing luciferase. Masuda-Nishimura et al. (2000) reported the use of a single tube (homogeneous) assay which employed a β-galactosidase substrate-modified luciferin.

Fluorescent or luminescent substrates or products of enzyme reactions have been employed in protein assay multiplexing. For example, fluorescent beads having ligands for up to 15 different cytokines were employed to detect two or more different cytokines (DeJager et al., 2003) and fluorescein diphosphate and casein BODIPY-FL were employed to detect alkaline phosphatase and certain proteases (Nolkrantz et al., 2002).

However, what is needed is an improved assay, e.g., a homogeneous assay, to detect two or more proteins using different detection techniques.

Summary of the Invention

The invention provides multiplexing of nonluminogenic, e.g., fluorescent or colorimetric, and luminogenic assays, e.g., in the same well, to detect the amount (e.g., activity) or presence in a sample of one or more moieties, including cofactors for enzymatic reactions such as ATP, proteins (peptides or polypeptides) that bind to and/or alter the conformation of a molecule, e.g., proteins that modify or cleave a peptide or polypeptide substrate, or a molecule which is bound by and/or altered by a protein. As used herein, a "luminogenic assay" includes a reaction in which a first molecule, e.g., a peptide or polypeptide substrate for a first enzyme, the product of a reaction between the first molecule and an appropriate (first) protein, and/or a product of a reaction between a different protein and the product of the first reaction, is luminogenic. Thus, a luminogenic assay may directly or indirectly detect, e.g., measure, the amount or presence of a cofactor for a reaction, a molecule which is bound by and/or altered by a protein, or the protein. For instance, in one embodiment, a beetle luciferase and an appropriate luciferin substrate may be employed in a luminogenic assay to detect ATP concentration, while in another embodiment a substrate for a luciferase, which is modified to contain a protease recognition site (modified, for example, via a covalent bond), may be employed in a luminogenic assay to detect the protease, i.e., when luciferase is present. Luminogenic assays include chemiluminescent and bioluminescent assays including but not limited to those which employ or detect luciferase, β-galactosidase, β-glucuronidase, β-lactamase, a protease, alkaline phosphatase, or peroxidase, and

suitable corresponding substrates, e.g., modified forms of luciferin, coelenterazine, luminol, peptides or polypeptides, dioxetanes, dioxetanones, and related acridinium esters. As used herein, a "luminogenic assay reagent" includes a substrate, as well as a cofactor(s) or other molecule(s) such as a protein, e.g., an enzyme, for a luminogenic reaction. In one embodiment, the luminogenic assay reagent may be Z-DEVD-aminoluciferin, Z-LETD-aminoluciferin, Z-LEHD-aminoluciferin, or may be other substrates, e.g., peptide or polypeptide substrates, linked to aminoluciferin, dihydroluciferin, luciferin 6' methylether, or luciferin 6' chloroethylether. A luminogenic assay is one in which a luminogenic reaction yields at least 1%, e.g., at least 10%, more light than a corresponding nonluminogenic assay.

A "nonluminogenic assay" includes a reaction in which a first molecule, e.g., a protein (a peptide or polypeptide), a (first) product of a reaction between the first molecule and a suitable (first) protein (peptide or polypeptide), or a product of a reaction between a different protein and the first product is/are not luminogenic but may be otherwise detectable, e.g., the substrate and/or product(s) are detected using a fluorescent or colorimetric assay, which directly or indirectly measures the amount or presence of a cofactor for the reaction, the molecule or the protein which interacts with the molecule. For instance, a substrate for an enzyme may be modified to contain a fluorophore that emits light of a certain wavelength only after the enzyme reacts with the substrate and the fluorophere is contacted with light of a certain wavelength or range of wavelengths, e.g., $(Z\text{-DEVD})_2$-rhodamine-110 is a substrate for a caspase, and cleavage of that substrate by the caspase may be monitored via fluorescence of rhodamine-110. As used herein, a "fluorogenic assay reagent" includes a substrate, as well as a cofactor(s) or other molecule(s), e.g., a protein, for a fluorogenic reaction. A nonluminogenic assay is one in which a nonluminogenic reaction yields less than about 10%, e.g., less than about 1% or less, the luminescent signal of a corresponding luminogenic assay.

In one embodiment, molecules employed in the assays of the invention, e.g., those which bind and/or are altered by a protein, include ones that are modified to contain a reporter molecule, i.e., a molecule which is detectable or capable of detection, e.g., after one or more subsequent reactions. For example, in one

embodiment, a substrate employed in a luminogenic assay of the invention includes a substrate for an enzyme to be detected, which substrate is covalently linked to a substrate for a luminogenic reaction, while in another embodiment a substrate employed in a fluorogenic assay may include a substrate for an enzyme to be detected, which substrate is covalently linked to one or more fluorophores. In some embodiments, the molecule which is bound by and/or altered by a protein does not contain a reporter molecule.

As described herein, the amount or presence of more than one protease in a sample was detected using at least two different substrates, one which had a luminescent readout and one or more of which had a fluorescent readout. For example, detection of a low abundance cellular protease was achieved using a more sensitive luminescent approach, e.g., detection of caspase-8 with the substrate Z-LETD-aminoluciferin, followed by a detection of another protease using another substrate, for instance, caspase-3 with $(Z\text{-}DEVD)_2$-rhodamine-110. This assay thus combines the strengths of both a fluorogenic reagent and the sensitivity of a luciferase-mediated luminescent reaction. Moreover, surprisingly, the presence of a luciferin, a molecule which has fluorescent properties and is often present in relatively large quantities in luminescent assays, did not result in significant interference in combined fluorescent/luminescent assays. Further, surprisingly, two caspases and a luciferase were detected in the same reaction mix, a mix which included a caspase-8 substrate (Z-LETD-aminoluciferin) and two caspase-3 substrates, i.e., $(Z\text{-}DEVD)_2$-rhodamine-110 and Ac-DEVD-AMC. The present invention thus provides more flexibility in molecules to be employed in multiplex assays, e.g., substrates for a luminogenic assay in combination with substrates for a fluorogenic assay. Moreover, if two enzyme-mediated reactions have compatible reagent conditions, the assay can be a one-step assay.

Accordingly, a combined luminogenic/nonluminogenic assay format of the present invention allows multiplexing of assays for one or more peptides or polypeptides, e.g., enzymes, one or more molecules which are bound by and/or altered by the peptide(s) or polypeptide(s), e.g., a peptide or polypeptide substrate for each enzyme, and/or one or more cofactors for each assay, or a combination

thereof. Thus, in one embodiment, the invention provides a method to detect the presence or amount of a first molecule for a first enzyme-mediated reaction and the presence or amount of a second molecule for a second enzyme-mediated reaction. The method includes contacting a sample suspected of having the first and/or second molecules with a reaction mixture for the first and second enzyme-mediated reactions which lacks the first and/or second molecules. The presence or amount of the first and the second molecules is then detected. The use of multiplexing which includes a luminescent assay provides increased sensitivity for the molecule detected using the luminescent assay. Thus, in one embodiment, a reaction mediated by the first enzyme yields a luminogenic product, whereas a reaction mediated by the second enzyme yields a nonluminogenic product. In one embodiment, a combined luminogenic/fluorogenic assay is provided including one in which one of the assays provides an internal control. The assays described herein may be employed with other assays, including reporter assays, nucleic-acid based assays or immunological-based assays and other unrelated enzyme assays.

The invention also provides a method for measuring the activity or presence of at least one molecule in a sample. The method includes providing a sample that may contain at least one molecule for an enzyme-mediated reaction, e.g., the sample may contain the enzyme, and contacting the sample with a reaction mixture for the enzyme-mediated reaction which lacks the molecule, e.g., the reaction mixture contains a substrate for the enzyme, so as to yield a reaction mixture wherein the presence or amount of the molecule is capable of being detected by a luminogenic assay. In one embodiment, the sample and/or reaction mixture is also contacted with reagents to detect a molecule for a second enzyme-mediated reaction, where the presence or amount of the molecule for the second enzyme-mediated reaction is capable of being detected by a nonluminogenic assay.

In one embodiment, the invention provides a method to detect the presence or amount of a first enzyme and/or a cofactor for a reaction mediated by that enzyme in a sample. The method includes contacting the sample with a first substrate for the first enzyme, a second substrate for a second enzyme, and optionally a third enzyme, to yield a reaction mixture. In one embodiment, at least

the first and second enzymes are not the same, e.g., do not substantially recognize the same substrate, i.e., they do not bind to the same substrate, or if they bind to and react with the same substrate, one of the enzymes does not react with a substrate for the other enzyme to the same extent (efficiency), i.e., one of the enzymes does not react substantially with a substrate for the other enzyme when substrates for both enzymes are present. As used herein, an enzyme (first enzyme) which does not react substantially with a substrate for a second enzyme includes an enzyme which, in a reaction having the second enzyme and equal amounts of a substrate for the first enzyme and a substrate for the second enzyme, cross reacts with the substrate for the second enzyme no more than 25%, e.g., cross reacts 15%, 10% or 5% or less, relative to a reaction between the first enzyme and substrate for the first enzyme. The first substrate, a product of a reaction between the first substrate and the first enzyme, and/or a product of a reaction between the third enzyme and the product of the first enzyme and the first substrate, is/are luminogenic. The second substrate, a (second) product of a reaction between the second substrate and the second enzyme, and/or a product of a reaction between another enzyme and the second product, is/are not luminogenic but otherwise detectable. The presence or amount of the first enzyme and/or cofactor is detected or determined. In one embodiment, the presence or amount of the second enzyme and/or a cofactor for the reaction mediated by the second enzyme is also detected or determined. In one embodiment, at least the first and second enzymes are not the same. The enzymes to be detected may be native enzymes or recombinant enzymes, e.g., including fusion proteins. The optional enzyme(s) added to the sample likewise may be native or recombinant enzymes.

In another embodiment, the invention provides a method to detect the presence or amount of a first enzyme and/or a cofactor for a reaction mediated by that enzyme in a sample. The method includes contacting the sample with a first substrate for the first enzyme, a second substrate for a second enzyme, and optionally a third enzyme, to yield a reaction mixture, wherein optionally at least the first and second enzymes are not the same. The first substrate, a product of a reaction between the first substrate and the first enzyme, and/or a product of a reaction between the third enzyme and the product of the first enzyme and the first

substrate, is/are not luminogenic but otherwise detectable. The second substrate, a second product of a reaction between the second substrate and the second enzyme, and/or a product of a reaction between another enzyme and the second product, is/are luminogenic. The presence or amount of the first enzyme and/or cofactor is detected or determined. In one embodiment, the presence or amount of the second enzyme is also detected or determined. The enzymes to be detected or employed in the reaction mixture may be native enzymes or recombinant enzymes.

Further provided is a method of assaying an enzyme-mediated luminescence reaction to detect a first enzyme or cofactor for a reaction mediated by that enzyme. The method includes contacting a sample with a first substrate for the first enzyme, a second substrate for a second enzyme, and optionally a third enzyme, to yield a reaction mixture, wherein the first and second enzymes are not the same. The first substrate, a product of the reaction between the first substrate and the first enzyme, and/or a product of the third enzyme and the product of the first enzyme and first substrate, is/are luminogenic. The second substrate, a second product of the reaction between the second substrate and the second enzyme, and/or a product of a reaction between the second product and another enzyme is/are not luminogenic but otherwise detectable. Luminescence is then detected. The method may further include detecting the presence or amount of the second enzyme, e.g., by detecting the presence or amount of the nonluminogenic substrate or product(s). In one embodiment, the second enzyme does not bind to or react with the first substrate, while in another embodiment, the first enzyme does not bind to or react with the second substrate. In one embodiment, at least the first and second enzymes are not the same. The enzymes to be detected or employed in the reaction mixture may be native enzymes or recombinant enzymes.

Also provided is a method of assaying an enzyme-mediated luminescence reaction to detect a first enzyme or cofactor for a reaction mediated by that enzyme. The method includes contacting a sample with a first substrate for the first enzyme, a second substrate for a second enzyme, and a third enzyme, to yield a reaction mixture. The first substrate, a product of the reaction between the first substrate and the first enzyme, and/or a product of the third enzyme and the product of the first

enzyme and first substrate, is/are not luminogenic but otherwise detectable. The second substrate, a second product of the reaction between the second substrate and the second enzyme, and/or a product of a reaction between the second product and another enzyme is/are luminogenic. Luminescence is then detected. The method may further include detecting the presence or amount of the first enzyme or product of the first enzyme and first substrate. In one embodiment, the second enzyme does not bind to or react substantially with the first substrate, while in another embodiment, the first enzyme does not bind to or react substantially with the second substrate. In one embodiment, at least the first and second enzymes are not the same. The enzymes to be detected or employed in the reaction mixture may be native enzymes or recombinant enzymes, e.g., including fusion proteins.

Further provided is a method to detect the presence or amount of at least two molecules in a sample. The method includes contacting a sample with a first substrate for a first enzyme, a second substrate for a second enzyme, and optionally a third enzyme, to yield a reaction mixture, wherein at least the first and second enzymes are not the same. A reaction between the first enzyme and the first substrate or the third enzyme and a product of the reaction between the first substrate and the first enzyme yields a luminogenic product. The second substrate, a second product of the reaction between the second substrate and the second enzyme, and/or a product of a reaction between the second product and a different enzyme, is/are not luminogenic. The presence or amount of the first and second enzymes and/or cofactor(s) is then detected. In one embodiment, luminescence is employed to detect the first enzyme and/or cofactor and fluorescence or colorimetry is employed to detect at least one other enzyme and/or cofactor. In one embodiment, substrates for two different enzymes are simultaneously combined with a sample to yield a reaction mixture. A reaction between one of the substrates and one of the enzymes directly or indirectly generates a luminescent signal while a reaction between the other substrate and enzyme directly or indirectly generates a fluorescent signal. Following an incubation period, the fluorescent signal is employed to detect the presence or amount of one enzyme and/or cofactor and the luminescent signal is employed to detect the presence or amount of the other

enzyme and/or cofactor. Specific buffer conditions can vary with the enzymes and/or cofactor(s) being detected, and can be determined by one of skill in the art of *in vitro* assays, e.g., enzyme assays. Alternatively, the assay can be a two-step assay, with reagent adjustment between the first and second assays. For example, reagent adjustment can include addition of a quenching agent for the first reaction, and/or an enhancing agent for the second reaction.

In one embodiment, to detect the first enzyme or cofactor for the first enzyme-mediated reaction and the second enzyme or cofactor for the second enzyme-mediated reaction, the sample is simultaneously contacted with the first substrate and the second substrate. In another embodiment, the sample is contacted with the second substrate before the first substrate, or is contacted with the first substrate before the second substrate. In one embodiment, the third or different enzyme may be added with the one or more substrates, before the one or more substrates or after the one or more substrates.

In one embodiment, to detect the first substrate or cofactor for the first enzyme-mediated reaction and the second substrate or cofactor for the second enzyme-mediated reaction, the sample is simultaneously contacted with the first enzyme and the second enzyme. In another embodiment, the sample is contacted with the second enzyme before the first enzyme, or is contacted with the first enzyme before the second enzyme.

In one embodiment, to detect the first enzyme or cofactor for the first enzyme-mediated reaction and the second substrate or cofactor for the second enzyme-mediated reaction, the sample is simultaneously contacted with the first substrate and the second enzyme. In another embodiment, the sample is contacted with the second enzyme before the first substrate, or is contacted with the first substrate before the second enzyme. In one embodiment, to detect the first substrate or cofactor for the first enzyme-mediated reaction and the second enzyme or cofactor for the second enzyme-mediated reaction, the sample is simultaneously contacted with the first enzyme and the second substrate. In another embodiment, the sample is contacted with the second substrate before the first enzyme, or is contacted with the first enzyme before the second substrate.

The sample employed in the methods of the invention may be a cell lysate, an *in vitro* transcription/translation reaction, a supernatant of a cell culture, a physiological fluid sample, e.g., a blood, plasma, serum, cerebrospinal fluid, tears or urine sample, and may include intact cells. The cells, cell lysate, or supernatant may be obtained from prokaryotic cells or eukaryotic cells.

The invention also provides for simultaneous or sequential detection of the presence or amount of the first and second proteins, e.g., enzymes, or a cofactor(s) for a reaction mediated by at least one of those proteins, e.g., for concurrent reactions or for sequential reactions optionally without quenching one of the reactions or enhancing/accelerating one of the reactions. In one embodiment, first and second substrates are added to the sample simultaneously and the amount or presence of the first enzyme and/or cofactor is detected before the amount or presence of the second enzyme and/or cofactor is detected. In another embodiment, the first and second substrates are added to the sample simultaneously and the presence or amount of the second enzyme and/or cofactor is detected before the amount or presence of the first enzyme and/or cofactor is detected. Alternatively, the first and second substrates are added to the sample simultaneously and the presence or amount of the first and second enzymes and/or cofactors is detected simultaneously. Preferably, the presence or amount of enzymes and/or cofactors are detected in a single reaction, e.g., all reactions are conducted in a single receptacle, e.g., well.

In another embodiment, the invention provides a method to detect the presence or amount of a molecule for an enzyme-mediated reaction in conjunction with expression of a fluorescent protein, e.g., green fluorescent protein. For example, cells which transiently or stably express a fluorescent protein, or a protein that can be labeled in cells to become fluorescent, such as dehalogenase, can be assayed for the presence or amount of the fluorescent protein via a fluorogenic assay as well as assayed for at least one additional molecule, e.g., an enzyme, substrate or co-factor for a reaction mediated by the enzyme, which molecule is present in or secreted by the cells via a luminogenic assay. In one embodiment, the presence or amount of a different molecule is also detected or determined, for example, in a

nonluminogenic assay. The presence or amount of the molecule(s) may then be normalized using data generated from the fluorescent protein.

Thus, the invention provides a method to detect the presence or amount of a molecule for a reaction mediated by a first enzyme. The method includes contacting a sample which comprises cells which express a fluorescent protein with a reaction mixture for the first enzyme which lacks the molecule, and optionally a second enzyme. A reaction mediated by the first enzyme yields a luminogenic product. The presence or amount of the molecule and the presence or amount of the fluorescent protein are then detected.

In one embodiment, for luminogenic and/or fluorogenic assays which yield products with different characteristics, e.g., different colors, further multiplexing (i.e., with other substrates) may be employed. For example, further multiplexing may include using different colors emitted by different luciferase based reactions or substrates or a fluorogenic assay with different excitation/emission spectra.

Also provided are kits which include one or more reagents for use in the assays of the invention.

The assay also has use as a drug discovery tool. Many drug-testing compounds have fluorescent properties that may interfere with a fluorescent/luminescent multiplex assay. The present invention provides assays to detect false results. As described herein, the same consensus substrate sequence for caspase-3 was linked to different reporter molecules with distinct spectral readouts, e.g., two with a fluorescent readout and one with a luminescent readout. Caspase-3 and luciferase were assayed in the presence and the absence of a caspase-3 inhibitor or a luciferase inhibitor. The data showed that there was very little interference between the three reporter molecules, and that luciferase could be used in a normalizing assay to control for false results.

Thus, the presence or amount of a modulator, for instance, an inhibitor, of an enzyme may be detected using a multiplex assay of the invention, e.g., a combined fluorogenic/luminogenic assay. In one embodiment, the method includes providing a reaction mixture comprising a nonluminogenic substrate for a first enzyme, a second substrate for the first enzyme, a second enzyme for a luminogenic assay, and

a test agent. A reaction between the nonluminogenic substrate and the first enzyme but not the second substrate and the first enzyme yields a nonluminogenic product, and a reaction between the second substrate and the first enzyme yields a substrate for the second enzyme, e.g., a substrate for a luciferase. A reaction between the substrate for the second enzyme and the second enzyme yields a luminogenic product. The presence or amount of the luminogenic product and the nonluminogenic product is compared in test and control reactions. Comparison of the two results indicates the effect of the modulator on the enzyme for the luminogenic assay, which can eliminate false results.

Brief Description of the Drawings

Figures 1A-B. Multiplex assay measuring the enzyme activities of caspase-3 and caspase-8 in the presence of both luminogenic and fluorogenic assay reagents. A) Relative light units (RLU) versus time. B) Relative fluorescence units (RFU) over time.

Figures 2A-C. Multiplex assay of caspase-3 and caspase-8. A) Signal to background fluorescence for AMC. B) Signal to background fluorescence for rhodamine-110. C) Signal to background luminescence.

Figures 3A-C. Triplex assay measuring the activities of caspase-3, caspase-8, and trypsin. A) RFU for rhodamine-110; B) RFU for AMC; C) RLU.

Figures 4A-D. Multiplex assay measuring a protease (caspase-3) and a non-protease (β-galactosidase) enzyme. A) and C), RLU at 1/2 hour and 18 hours, respectively. B) and D) RFU at 2 hours and 18 hours, respectively.

Figures 5A-C. Excitation and emission spectra of luciferin (A), aminoluciferin (B) and Z-LETD-aminoluciferin (C).

Figure 6. Signal to background ratios in three channels; rhodamine-110, AMC and luminescence, in a caspase-3 assay.

Figures 7A-D. Multiplex fluorogenic and luminogenic assays measuring lactate dehydrogenase (LDH) activity and adenosine triphosphate (ATP). A) and C) RLU versus ATP concentration. B) and D) RFU versus LDH dilution.

Figures 8A-D. Multiplex fluorogenic and luminogenic assays measuring LDH and caspase-3. A) and C) RLU versus caspase-3 concentration. B) and D) RFU versus LDH dilution.

Figures 9A-D. Multiplex fluorogenic and luminogenic assays measuring protein kinase A (PKA) and caspase-3. A) and C) RLU versus caspase-3 concentration. B) and D) RFU versus PKA concentration.

Figure 10. Multiplex fluorogenic and luminogenic assays measuring caspase-3 and *Renilla* luciferase (luc). A) and C) RLU versus staurosporine concentration. B) and D) RFU versus caspase-3 concentration.

Detailed Description of the Invention

The invention provides a multiplexed assay method in which at least two different molecules which bind to and/or are altered by a protein (e.g., peptide or polypeptide) are provided either simultaneously or sequentially in a reaction mixture to detect one or more moieties including proteins (peptides or polypeptides), e.g., enzymes, or substrates or cofactors for reactions. For instance, one or more enzyme-mediated reactions are performed under conditions effective to convert at least one enzyme substrate to a product of a reaction between the substrate and the enzyme. Preferably, each molecule in the reaction mixture, e.g., substrate, or product in the reaction has a different characteristic from other molecule(s) or product(s), and, in one embodiment, at least one molecule includes a reporter molecule capable of directly or indirectly producing a detectable signal. The resulting signal is related to the presence or amount of the molecule to be detected. In one embodiment, the method includes performing two or more enzyme reactions in the presence of at least two different enzyme substrates under conditions effective to convert each substrate to a corresponding product, where at least the substrate or product of each reaction, and/or a product of a reaction between one of the products and a third, e.g., different, enzyme, has a different detectable characteristic, e.g., a different optical characteristic, from the other substrate(s) and/or product(s). After performing the reactions, either simultaneously or sequentially, the presence or amount of one or more substrates or one or more products of the reaction(s) is/are

detected or determined. From this, the presence or amount of the corresponding enzyme(s) and/or cofactors can be determined.

Thus, two general types of multiplexed assays are contemplated. In the first, multiple moieties, e.g., one or more enzymes, one or more substrates and/or one or more cofactors for an enzyme-mediated reaction, are assayed in the same reaction mixture. Each enzyme is capable of converting at least one of the substrates to a corresponding product, where the substrate(s) and/or corresponding product(s), or product(s) of a reaction between one of the corresponding products and another enzyme, have different detectable characteristics that allow the substrates and/or the products to be individually detected when present in the same reaction mixture. The order of adding the molecules for the assays of the present invention can vary. Thus, individual reactions may be initiated and/or conducted simultaneously or sequentially. If initiated and conducted sequentially, the different detectable characteristics may require different detection methods, and/or adjustments to reaction conditions, e.g., reagent concentration, temperatures or additional reagents, may be performed. For instance, a quenching agent or enhancing agent may be added between reactions (see, e.g., U.S. Patent Nos. 5,774,320 and 6,586,196, the disclosures of which are specifically incorporated by reference herein). In one preferred embodiment, the two or more reactions are carried out simultaneously in a single reaction mixture, where each of the enzymes is effective to convert one of the substrates in the reaction mixture to a product. This embodiment may be used, for example, to determine the presence or amount of at least two different enzymes and/or cofactors in a cell, cell lysate or cell supernatant. In addition, the reaction may contain one or more test agents, e.g., enzyme inhibitors or activators, and/or different concentrations of inhibitors, activators, or substrates.

Optionally, the assays are employed as a homogeneous assay, e.g., the one or more substrates and additional components are mixed prior to adding the mixture to the sample. Results may be read without additional transfer of reagents.

In a second assay type, two or more enzyme-mediated reactions are carried out in tandem. The separate reactions may be performed at the same time or at different times. The reactions may contain one or more of the same or different

enzymes, one or more of the same or different test agents, e.g., enzyme inhibitors or activators, and/or different concentrations of inhibitors, activators, or substrates. In one embodiment, each reaction mixture contains at least two substrates capable of being converted to a product, where the substrate(s) and/or corresponding product(s), and/or a product(s) of a reaction between the product of one of the enzyme/substrate pairs and a different enzyme, have different detectable characteristics.

The assays of the present invention thus allow the detection of multiple enzymes or cofactors in a sample, e.g., a sample which includes eukaryotic cells, e.g., yeast, avian, plant, insect or mammalian cells, including but not limited to human, simian, murine, canine, bovine, equine, feline, ovine, caprine or swine cells, or prokaryotic cells, or cells from two or more different organisms, or cell lysates or supernatants thereof. The cells may not have been genetically modified via recombinant techniques (nonrecombinant cells), or may be recombinant cells which are transiently transfected with recombinant DNA and/or the genome of which is stably augmented with a recombinant DNA, or which genome has been modified to disrupt a gene, e.g., disrupt a promoter, intron or open reading frame, or replace one DNA fragment with another. The recombinant DNA or replacement DNA fragment may encode a molecule to be detected by the methods of the invention, a moiety which alters the level or activity of the molecule to be detected, and/or a gene product unrelated to the molecule or moiety that alters the level or activity of the molecule.

In one embodiment, the methods according to the present invention provide a rapid, highly sensitive method for simultaneously or sequentially detecting multiple moieties including enzymes in a single sample such as an aliquot of cells or a lysate thereof. In one embodiment, the method includes quantifying the presence or amount (activity) of a first enzyme, substrate or cofactor in a luminogenic assay and quantifying the presence or amount of a second enzyme, substrate or cofactor in a nonluminogenic assay, such as a fluorogenic assay. In one embodiment, reagents, e.g., substrates, for each reaction may be added together or sequentially. In another embodiment, the method includes quantifying the presence or amount of a first

enzyme, substrate or cofactor in a fluorogenic assay and quantifying the presence or amount of a second enzyme, substrate or cofactor in a luminogenic assay. Thus, in another embodiment, the method includes quantifying the presence or amount of a cofactor in a luminogenic assay and quantifying a different molecule in a nonluminogenic assay. In yet another embodiment, the method includes quantifying the presence or amount of a cofactor in a nonluminogenic assay and quantifying a different molecule in a luminogenic assay. The intensity of the luminogenic or nonluminogenic signal is a function of the presence or amount of the respective molecule.

In one embodiment, the present invention relates to a method of measuring the presence or amount of multiple enzymes in a single aliquot of cells or a lysate thereof. In one embodiment, at least one of the enzymes is an endogenous enzyme, For example, in one embodiment, the present invention provides an improved, sensitive method for monitoring the activity of at least one protease and optionally another enzyme in preparations comprising the protease and the other enzyme, including purified preparations from either prokaryotic or eukaryotic cells, cell lysates or supernatants of cells such as cultured eukaryotic cells, e.g., mammalian cells. For enzymes present in different cellular locations, such as a secreted and an intracellular protease, a substrate for each enzyme can be added to a well with intact cells. The presence or amount of the secreted protease may be detected prior to detection of the intracellular protease, such as after cell lysis, e.g., where the detection of the intracellular protease is in the same receptacle, for instance, same well, as that for the secreted protease. In one embodiment, a non-cell permeant substrate for an intracellular protease and a substrate for a secreted protease are added to a sample comprising cells and the cells are then lysed. Detection of the secreted protease may be before cell lysis or after cell lysis. In another embodiment, a non-cell permeant substrate for an intracellular enzyme or a secreted protease, and a cell permanent substrate for a second intracellular enzyme are added to a sample comprising cells. The presence of the second intracellular enzyme and the secreted protease may be detected without lysis. In yet another embodiment, a triplex assay is performed to detect a secreted protease, an intracellular enzyme (by employing

either a cell permeant substrate or non-cell permeant substrate) and a second intracellular enzyme (by employing either a cell permeant substrate or a non-cell permeant substrate). In one embodiment, the secreted protein is detected using fluorescence or spectrophotometry.

The present methods can be employed to detect any molecule including any enzyme or any set of enzymes. The enzymes employed in the methods, either enzymes to be detected or enzymes which are useful to detect a substrate or cofactor, can be selected from any combination of enzymes including recombinant and endogenous (native) enzymes. In one embodiment, all of the enzymes to be detected are endogenous enzymes. In another embodiment, two enzymes to be detected are endogenous enzymes and another enzyme is a recombinant enzyme. In another embodiment, one enzyme is an endogenous enzyme and another enzyme is a recombinant enzyme. Other combinations apparent to one of ordinary skill in the art can be used in the present assays and methods according to the teachings herein. The enzymes include but are not limited to proteases, phosphatases, peroxidases, sulfatases, peptidases, and glycosidases. The enzymes may be from different groups based on the nature of the catalyzed reaction, groups including but not limited to hydrolases, oxidoreductases, lyases, transferases, isomerases, ligases, or synthases, or they may be from the same group so long as at least one of the enzymes has a partially overlapping or preferably a substantially different substrate specificity relative to at least one of the other enzymes. Of particular interest are classes of enzymes that have physiological significance. These enzymes include protein kinases, peptidases, esterases, protein phosphatases, isomerases, glycosylases, synthetases, proteases, dehydrogenases, oxidases, reductases, methylases and the like. Enzymes of interest include those involved in making or hydrolyzing esters, both organic and inorganic, glycosylating, and hydrolyzing amides. In any class, there may be further subdivisions, as in the kinases, where the kinase may be specific for phosphorylation of serine, threonine and/or tyrosine residues in peptides and proteins. Thus, the enzymes may be, for example, kinases from different functional groups of kinases, including cyclic nucleotide-regulated protein kinases, protein kinase C, kinases regulated by Ca^{2+}/CaM, cyclin-dependent kinases,

ERK/MAP kinases, and protein-tyrosine kinases. The kinase may be a protein kinase enzyme in a signaling pathway, effective to phosphorylate an oligopeptide substrate, such as ERK kinase, S6 kinase, IR kinase, P38 kinase, and AbI kinase. For these, the substrates can include an oligopeptide substrate. Other kinases of interest may include, for example, Src kinase, JNK, MAP kinase, cyclin-dependent kinases, P53 kinases, platelet-derived growth factor receptor, epidermal growth factor receptor, and MEK.

In particular, enzymes that are useful in the present invention include any protein that exhibits enzymatic activity, e.g., lipases, phospholipases, sulphatases, ureases, peptidases, proteases and esterases, including acid phosphatases, glucosidases, glucuronidases, galactosidases, carboxylesterases, and luciferases. In one embodiment, one of the enzymes is a hydrolytic enzyme. In another embodiment, at least two of the enzymes are hydrolytic enzymes. Examples of hydrolytic enzymes include alkaline and acid phosphatases, esterases, decarboxylases, phospholipase D, P-xylosidase, β-D-fucosidase, thioglucosidase, β-D-galactosidase, α-D-galactosidase, α-D-glucosidase, β-D-glucosidase, β-D-glucuronidase, α-D-mannosidase, β-D-mannosidase, β-D-fructofuranosidase, and β-D-glucosiduronase.

A substrate or cofactor for any particular enzyme-mediated reaction is known to those of skill in the art. Exemplary cleavage sites for some proteases are set forth in Table 1.

Table 1

Protease	Cut Site(s)
Aminopeptidase M	Hydrolysis from free N-terminus
Carboxypeptidase Y	Hydrolysis from C-terminus
Caspase-1,4,5	W/LEHD-X
Caspase-2,3,7	DEXD-X
Caspase-6,8,9	L/VEXD-X
Chymotrypsin	Y-X, F-X, T-X, (L-X, M-X, A-X, E-X)
Factor Xa	IEGR-X
Pepsin	F-Z, M-Z, L-Z, W-Z (where Z is a hydrophobic residue) but will cleave others
TEV	E(N)XYXQ-S/G
Thrombin	R-X
Trypsin	R-X, K-X
Tryptase	PRNK-X
β-secretase	EISEVK/NM/L-DAEFRHD, e.g., SEVNL-DAEFR

X is one or more amino acids

For alkaline phosphatase, it is preferable that the substrate includes a phosphate-containing dioxetane, such as 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt, or disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'(5'-chloro)-tricyclo-[3.3.1.1^{3,7}]decan]-4-yl]phenyl phosphate, or disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(^{5}'-chloro)-tricyclo{3.3.1.13,7]decan}-4-yl)-1-phenyl phosphate or disodium 2-chloro-5-(^{4}-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1. 13,7]decan}-4-yl)-1-phenzyl phosphate (AMPPD, CSPD, CDP-Star® and ADP-Star™, respectively).

For β-galactosidase, the substrate preferably includes a dioxetane containing galactosidase-cleavable or galactopyranoside groups. The luminescence in the assay results from the enzymatic cleavage of the sugar moiety from the dioxetane substrate. Examples of such substrates include 3-(2'-spiroadamantane)-4-methoxy-4-(3"-β-D-galactopyranosyl)phenyl-1, 2-dioxetane(AMPGD), 3-(4-

methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.$1^{3,7}$]-decan]-4-yl-phenyl-β-D-galactopyranoside (Galacton®), 5-chloro-3-(methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.$1^{3,7}$]decan-4-yl-phenyl-β-D-galactopyranoside (Galacton-Plus®), and 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'(5'-chloro)-tricyclo-[3.3.1.$1^{3,7}$]decan]-4-yl)phenyl β-D-galactopyranoside (Galacton-Star®).

In assays for β-glucuronidase and β-glucosidase, the substrate includes a dioxetane containing β-glucuronidase-cleavable groups such as a glucuronide, e.g., sodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl-β-D-glucuronate (Glucuron™). In assays for a carboxyl esterase, the substrate includes a suitable ester group bound to the dioxetane. In assays for proteases and phospholipases, the substrate includes a suitable enzyme-cleavable group bound to the dioxetane.

Preferably, the substrates for each enzyme in the assay are different. For assays which include one dioxetane containing substrate, the substrate optionally contains a substituted or unsubstituted adamantyl group, a Y group which may be substituted or unsubstituted and an enzyme cleavable group. Examples of preferred dioxetanes include those mentioned above, e.g., those referred to as Galacton®, Galacton-Plus®, CDP-Star®, Glucuron™, AMPPD, Galacton-Star®, and ADP-Star™, as well as 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl-β-D-glucopyranoside (Glucon™), CSPD, disodium 3-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'(5'-chloro)-tricyclo-[3.3.1.$1^{3,7}$]decan)-4-yl)-1-phenyl phosphate (CDP).

Preferably, a substrate for at least one enzyme to be detected is modified to contain a reporter molecule. A reporter molecule is any molecule that allows a substrate linked to that molecule, a product resulting from a reaction between the enzyme and the substrate, or a product of a reaction between that product and another enzyme, to be differentially detected, preferably quantitatively. Reporter molecules include but are not limited to optic molecules such as fluorophores, an absorptive colored particle or a dye, radiolabels, enzymes such as a catalytic moiety that is effective to catalyze a detectable reaction in the presence of suitable reaction

components, a subunit or fragment of an enzyme that is functional when associated with other subunit(s) or fragment(s), or a substrate for a subsequent reaction, e.g., one in which the product of that reaction is detectable. As used herein, a "fluorophore" includes a molecule which is capable of absorbing energy at a wavelength range and releasing energy at a wavelength range other than the absorbance range. The term "excitation wavelength" refers to the range of wavelengths at which a fluorophore absorbs energy. The term "emission wavelength" refers to the range of wavelengths that the fluorophore releases energy or fluoresces.

In one embodiment, the reporter molecule fluoresces. One group of fluorescers is the xanthene dyes, which include the fluoresceins, rosamines and rhodamines. These compounds are commercially available with substituents on the phenyl group, which can be used as the site for bonding or as the bonding functionality. For example, amino and isothiocyanate substituted fluorescein compounds are available.

Another group of fluorescent compounds are the naphthylamines, having an amino group in the alpha or beta position, usually alpha position. Included among the naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-napththalene sulfonate and 2-p-toluidinyl-6-naphthalene sulfonate. Some naphthalene compounds are found to have some non-specific binding to protein, so that their use requires employing an assay medium where the amount of protein is minimized. Other fluorescers are multidentate ligands that include nitrogen-containing macrocycles, which have conjugated ring systems with pi-electrons. These macrocycles may be optionally substituted, including substitution on bridging carbons or on nitrogens. Suitable macrocycles include derivatives of porphyrins, azaporphyrins, corrins, sapphyrins and porphycenes and other like macrocycles, which contain electrons that are extensively delocalized. The azaporphyrin derivatives include phthalocyanine, benzotriazaporphyrin and naphthalocyanine and their derivatives.

In some instances fluorescent fusion proteins may be employed, e.g., a green, red or blue fluorescent protein or other fluorescent protein fused to a

polypeptide substrate. In other embodiments, a fluorescent protein may itself be a substrate for a hydrolytic enzyme. A "fluorescent protein" is a full-length fluorescent protein or a fluorescent fragment thereof.

A non-limiting list of chemical fluorophores of use in the invention, along with their excitation and emission wavelengths, is shown in Table 2. Excitation and emission values can change depending on reaction conditions, such as pH, buffer system, or solvent.

Table 2

Fluorophore	Excitation (nm)	Emission (nm)
Fluorescein (FITC)	495	525
Hoechst 33258	360	470
R-Phycoerythrin (PE)	488	578
Rhodamine (TRITC)	552	570
Quantum Red™	488	670
Texas Red	596	620
Cy3	552	570
Rhodamine-110	499	521
AFC	380	500
AMC	342	441
Resorufin	571	585
BODIPY FL	504	512
BODIPY TR	591	620

In one embodiment, one of the enzymes is detected using a substrate which includes an amino-modified luciferin or a carboxy protected derivative thereof, which modification includes a substrate for the enzyme. In one embodiment, the modification is one or more amino acid residues which include a recognition site for a protease. In one embodiment, the substrate is covalently linked to the amino group of aminoluciferin or a carboxy-modified derivative thereof via a peptide bond. Preferably, the N-terminus of a peptide or protein substrate is modified to prevent degradation by aminopeptidases, e.g., using an amino-terminal protecting group. In the absence of the appropriate enzyme or cofactor, a mixture including such a substrate and luciferase generates minimal light as minimal aminoluciferin is present. In the presence of the appropriate enzyme, the bond linking the substrate

and aminoluciferin can be cleaved by the enzyme to yield aminoluciferin, a substrate for luciferase. Thus, in the presence of luciferase, for instance, a native, recombinant or mutant luciferase, and any cofactors and appropriate reaction conditions, light is generated, which is proportional to the presence or activity of the enzyme.

In one embodiment, one of the enzymes is detected using a substrate which includes a fluorophore. In one embodiment, the substrate includes one or more amino acid residues which include a recognition site for a protease. In one embodiment, the substrate is covalently linked to one or more fluorophores. In the absence of the appropriate enzyme or cofactor, a mixture including such a substrate generates minimal light at the emission wavelength as the fluorescent properties of the fluorophore are quenched, e.g., by the proximity of the quenching group such that the properties of a substrate-fluorophore conjugate are changed, resulting in altered, e.g., reduced, fluorescent properties for the conjugate relative to the fluorophore alone. In the presence of the appropriate enzyme, cleavage of the conjugate yields the fluorophore. In another embodiment, prior to cleavage, the conjugate is fluorescent but after cleavage with the enzyme, the product(s) have altered spectra.

In one embodiment, the conditions for at least two of the reactions are compatible. For instance, the conditions for at least 2 enzymes, and preferably the conditions for 3 or more enzymes, e.g., 4 or more enzymes, are compatible. A group of similar enzymes will generally have compatible reaction conditions, such as pH and ionic strength, however, cofactor requirements, metal ion requirements, and the like, involving assay components having relatively low mass concentrations, e.g., cofactors, need not be common. Common conditions include conditions such that each of the enzymes provides a measurable rate during the course of the reaction and will generally be that each of the enzymes has at least about 10%, usually at least about 20%, preferably at least about 50%, of its maximum turnover rate for the particular substrate, without significant interference from the components added for the other enzyme(s).

Alternatively, the conditions for one reaction may not be compatible with another reaction although substrates for both reactions are present. In such embodiments, one enzyme is active but cannot react with its substrate. In one embodiment, for example, where conditions for two reactions are not compatible, individual enzyme-assay reactions are carried out sequentially and/or in separate reaction mixtures. Following the enzyme assay, the reaction mixture (or a portion thereof) may be combined with another reaction. Each individual reaction mixture may contain one or more enzymes and one or more substrates. In its simplest form, a single enzyme to be assayed and a single substrate for that enzyme are in each reaction mixture. The set of substrates employed in the reaction has the same general properties as that required in the single-reaction multiplexed assay. That is, each substrate and/or corresponding product have unique characteristics, allowing them to be distinguished from one another.

The order of detection of molecules in the reactions can vary. In one embodiment, regardless of whether reactions are initiated at the same time or not, the molecule detected by a luminogenic assay is detected, then the molecule detected by the nonluminogenic assay is detected. Alternatively, regardless of whether reactions are initiated at the same time or not, the molecule detected by the nonluminogenic assay is detected, then the molecule detected by the luminogenic assay is detected. In other embodiments, the presence or amount of two or more molecules is detected essentially simultaneously. In one embodiment, the presence or activity of one molecule to be detected is substantially decreased prior to detecting the presence or activity of the second molecule, e.g., by waiting until the first signal has diminished, e.g., by at least 50%, or by adding a quenching agent for the first reaction. Thus, in some embodiments, one or more of the reactions are terminated, e.g., by inhibiting an enzyme for the reaction, prior to detection. Preferably, the signal produced by one assay does not substantially interfere with the quantification of the signal produced by at least one other assay.

The present invention also provides kits for detecting the presence or activity of one or more peptides or proteins, molecules which bind to and/or are altered by the peptides or proteins, or cofactors in a sample such as a sample including intact

cells, a cell lysate, e.g., a lysate which is at least partially purified, or a cellular supernatant. Such a kit includes at least one reagent for quantifying at least one of the peptides and/or proteins, molecules bound by and/or altered by the peptides and/or proteins, or cofactors, such as a substrate for at least one enzyme.

The invention will be further described by the following non-limiting examples. For all examples, suitable control reactions are readily designed by those skilled in the art.

Example I

Fluorescent/Luminescent Multiplex Assays

A. Measurement of caspase-3 and caspase-8 in a single well, multiplex assay

Caspase-Glo™ 8 Reagent (Caspase-Glo™ 8 Assay System, Promega, Corp.) was evaluated for its ability to allow multiplexing of homogeneous luminogenic caspase-8 and nonluminogenic caspase-3 enzyme assays. Caspase-Glo™ 8 Reagent is comprised of Caspase-Glo™ 8 Buffer and the luminogenic substrate Z-LETD-aminoluciferin. For the luminogenic assays in Figure 1A, either Caspase-Glo™ 8 Reagent (diamonds) or Caspase-Glo™ 8 Reagent also containing 50μM of the fluorogenic substrate for caspase-3, $(Z\text{-}DEVD)_2$-rhodamine-110 (squares), was used to demonstrate the feasibility of a multiplexed luminogenic and nonluminogenic assay. For the fluorogenic assay in Figure 1B, Caspase-Glo™ 8 Buffer containing either 50 μM $(Z\text{-}DEVD)_2$-rhodamine-110 and 10 mM DTT (diamonds) or 50 μM $(Z\text{-}DEVD)_2$-rhodamine-110 and Z-LETD-aminoluciferin (squares) were used.

Dilutions of caspase-8 enzyme, caspase-3 enzyme, and combined caspase-8 and caspase-3 enzymes (Biomol Research Laboratories) were prepared in RPMI 1640 (Sigma Corporation) to a final concentration of 100 units/ml. 100 μl of caspase-8 dilutions, a mixture of caspase-8 and caspase-3 dilutions, or caspase-3 dilutions, were added to separate wells of a 96-well plate. 100 μl of Caspase-Glo™ 8 Reagent with or without 50 μM $(Z\text{-}DEVD)_2$-rhodamine-110 (Figure 1A), or 100 μl of Caspase-Glo™ 8 Buffer supplemented with $(Z\text{-}DEVD)_2$-rhodamine 110 and

DTT with or without Z-LETD-aminoluciferin (Figure 1B) were added to reach a final volume of 200 μl/well. The reaction plate was incubated at room temperature for at least ten minutes on a plate shaker.

After incubation, relative luminescence was determined using a DYNEX Laboratories MLX™ plate luminometer, and relative fluorescence was measured with a CytoFluor II Fluorescent plate reader outfitted with a $485_{EX}/530_{EM}$ filter set.

Results

The simultaneous measurement of fluorescence and luminescence for two protease enzymes in a single well is shown in Figure 1. As seen in Figure 1A, the presence of caspase-3 and its fluorogenic substrate, (Z-DEVD)$_2$-rhodamine-110, in a luminogenic assay for caspase-8 (squares) does not greatly alter the luminescent reaction. Similarly, as seen in Figure 1B, the presence of caspase-8 and its luminogenic substrate Z-LETD-aminoluciferin in a fluorogenic assay for caspase-3 (squares) does not impact the fluorogenic assay for caspase-3.

B. Background determinations for a caspase-3 and caspase-8 multiplex assay

Various concentrations of luminogenic and fluorogenic reagents, including caspase enzymes and substrates thereof, and buffer components were combined to establish each constituent's contribution to fluorescence and/or luminescence. The fluorogenic substrate (Z-DEVD)$_2$-rhodamine-110 reports caspase-3 activity in the rhodamine channel ($485_{EX}/520_{EM}$) and the fluorogenic substrate Ac-DEVD-AMC reports the caspase-3 activity in the AMC channel ($360_{EX}/460_{EM}$), while the substrate Z-LETD-aminoluciferin reports caspase-8 activity during luminescence measurement. Table III describes the amount of each component (μl) for twelve different reaction conditions resulting in a total volume of around 500 μl of master mix, or 100 μl of master mix/reaction (n=4) for each reaction condition. For the 'caspase added' row, the number in this row defines the type of caspase added in overabundance and does not describe a volume.

Table III

Component	1	2	3	4	5	6	7	8	9	10	11	12
Caspase-Glo™ 8 Buffer	400	400	400	400	400	400	400	400	400	400	400	495
Caspase-Glo™ 8 lyophilized substrate reconstituted in 1ml of water	100	na	na	100	100	100	100	100	100	100	100	na
5 mM $(Z\text{-}DEVD)_2$-rhodamine-110	na	5	na	5	5	na	na	na	5	5	na	5
5 mM Ac-DEVD-AMC	na	na	5	na	na	5	na	na	na	na	na	na
DMSO	5	na	na	na	na	na	5	5	na	na	5	na
100 mM Hepes	na	80	80	na	na	na	na	na	na	na	na	na
1 M DTT	na	20	20	na	na	na	na	na	na	na	na	na
Caspase-3 inhibitor (in excess)	na	na	na	na	Yes	na	na	Yes	na	na	Yes	na
Caspase added	8	3	3	8&3	8&3	8&3	3	3	8	3	8	3

1. Caspase-8 luc control
2. Caspase-3 rhodamine-110 control
3. Caspase-3 AMC control
4. Multiplex control with rhodamine-110
5. Multiplex control with rhodamine-110 + inhibitor
6. Multiplex control with AMC
7. Capase-3 with aminoluciferin mix
8. Caspase-3 with aminoluciferin mix + inhibitor
9. Caspase-8 with rhodamine-110
10. Caspase-3 with aminoluciferin mix
11. Caspase-8 with aminoluciferin mix + inhibitor

12. Caspase-3 with rhodamine-110 without aminoluciferin mix

The components from Table III were added to replicate wells and reactions were incubated at room temperature for two hours. The buffer employed was that from the Caspase-Glo™ 8 Assay System. DMSO was obtained from Sigma-Aldrich and the DTT was obtained from Amresco. The substrates and inhibitors were obtained from Promega Corp.

Relative luminescence was determined using a DYNEX Laboratories MLX™ plate luminometer. Fluorescence was determined using a CytoFluor II Fluorescent plate reader outfitted with a $485_{EX}/530_{EM}$ filter set for rhodamine-110 and then $360_{EX}/460_{EM}$ for the AMC channel.

Results

For Figures 2A, B, and C, all carats represent where either fluorescence or luminescence indicating enzyme activity was expected. Figure 2A shows the signal for AMC fluorescence in each reaction. Fluorescence above background was only present where the appropriate substrate/enzyme combination of Ac-DEVD-AMC and caspase-3 was present (reaction conditions 3 and 6). Figure 2B shows the signal for rhodamine-110 fluorescence in each reaction. Fluorescence above background was present where the substrate/enzyme combination of $(\text{Z-DEVD})_2$-rhodamine-110/caspase-3 was present (reaction conditions 2, 4, 10, and 12), except when a caspase-3 inhibitor was present (reaction condition 5). For luminescence signal above background (Figure 2C), those reactions with the appropriate substrate/enzyme combination of Z-LETD-aminoluciferin/caspase-8 showed signal above background (reaction conditions 1, 4, 6, 7, 9, and 10), except those reaction conditions where a caspase inhibitor was present (reaction conditions 5, 8, and 11). The data thus demonstrate that there was negligible contribution of reaction components to background fluorescence and luminescence measurements under these conditions.

C. Measurement of caspase-3, caspase-8, and trypsin in a single well, triplex assay

Dilutions of detectable levels of caspase-8 (150 units/ml, Biomol Research Laboratories), caspase-3 (Pharmingen Corp.), trypsin (Sigma Corp.), and a combination of all three enzymes, were prepared in Dulbecco's phosphate buffered saline (Sigma Corp.). 100 μl of each enzyme dilution were added to the wells of a

96-well plate and 100 μl of each substrate, either singly or in combination as appropriate, were added to the corresponding wells: substrate (Z-DEVD)$_2$-rhodamine-110 for caspase-3, substrate Z-PRNK-AMC for trypsin (as described in U.S. patent application Serial No. 09/955,639 as a substrate for beta-tryptases but with a recognized lesser utility for trypsin, incorporated herein in its entirety), and substrate Z-LETD-aminoluciferin for caspase-8. When Caspase-Glo™ 8 Buffer was employed with a substrate for a fluorogenic assay, 10 mM DTT was included. Plates were incubated for at least ten minutes at room temperature on a plate shaker.

Following incubation, relative luminescence for caspase-8 activity was measured using BMG Fluorostar (BMG Labtechnologies Ltd.). Relative fluorescence was determined using the Labsystems Fluoroskan Ascent plate reader. For caspase-3 activity, a filter set of $485_{EX}/527_{EM}$ was utilized. For trypsin activity, a filter set of $360_{EX}/460_{EM}$ was used.

Results

As shown in Figure 3A, the conditions employed to detect caspase-3 in a reaction with three different substrates and corresponding enzymes combined (the triplex assay) yielded relatively high fluorescence over that of the control conditions. When comparing the activity of caspase-3 in the triplex assay (all substrates with all enzymes) to that of caspase-3 alone, caspase-3 activity was greater than background when caspase-3 was in the same reaction with the other triplex enzyme reactions. Similar results were seen for trypsin (Figure 3B) and caspase-8 (Figure 3C), albeit not to the same extent as with caspase-3.

D. Measurement of caspase-3 and β-galactosidase in a single well, multiplex format

Reagents were prepared by reconstituting Beta-Glo® lyophilized substrate with Beta-Glo® Buffer (Beta-Glo® Assay System, Promega Corp.), or adding (Z-DEVD)$_2$-rhodamine-110 (50 μM) to Beta-Glo® Buffer, or reconstituting Beta-Glo® lyophilized substrate with Beta-Glo® Buffer and adding (Z-DEVD)$_2$-rhodamine-110 (50 μM). Caspase-3 (2 μl/ml, Pharmingen Corp), or β-galactosidase (0.1 μl/ml), or caspase-3 and β-galactosidase, were diluted in RPMI 1640 and 100 μl were added to wells of a 96-well white plate. 100 μl of the appropriate reagent were added to wells of a 96-well plate and the plates were incubated at room temperature.

Luminescence was measured using a DYNEX Laboratories MLX™ plate luminometer at 30 minutes. Fluorescence was measured 2 hours post incubation on a CytoFluor II Fluorescent plate reader with a filter set of $485_{EX}/530_{EM}$. All measurements were repeated at 18 hours with different gain settings on the CytoFluor II fluorometer to compensate for increased fluorescence.

Results

Figures 4A and C demonstrate the luminogenic assay for ß-galactosidase is functional in the presence of the fluorogenic reagent to measure caspase-3. Figures 4B and D demonstrate the fluorogenic assay to measure caspase-3 is functional in the presence of the luminogenic reagent to measure ß-galactosidase. As seen in Figures 4B and 4D, there was a minor contribution of the luminogenic reagent components to background fluorescence. However, there was almost no contribution of the fluorogenic reagent components to luminescence (Figures 4A and 4C).

E. Spectral scans of substrates for luminogenic assays

Luciferin, aminoluciferin, and Z-LETD-aminoluciferin, were diluted to approximately 2 μM in a buffer containing 0.1M Tris pH 7.3, 2 mM EDTA, and 10 mM $MgSO_4$. Samples were scanned on a SPEX Fluorolog-2 spectrometer with 1.25 mm excitation and emission slit filter present, at 1 nm wavelength interval and 0.2 second integration time. All scans were performed using a quartz cuvette.

Results

For luciferin and aminoluciferin, excitation was at 325 nm and emission was captured from 375 to 750 nm, and excitation was captured at 280-550 nm with emission measured at 600 nm (Figures 5A and 5B). For Z-LETD-aminoluciferin, excitation was at 325 nm and emission was captured between 375-750 nm, and excitation was captured at 280-500 nm with emission measured at 525 nm (Figure 5C). Interestingly, when a peptide was conjugated to aminoluciferin (Figure 5C), the emission peak of the conjugate was blue shifted to shorter wavelengths. This was unexpected and therefore allows for dual luminscence/fluorescent measurements, particularly when using a fluorophore that emits in the same wavelength range as aminoluciferin emits.

Example II

Method to Detect False Results

Methods

Caspase-Glo™ 3/7 Reagent (Caspase-Glo™ 3/7 Assay, Promega, Corp.) which contains Z-DEVD-aminoluciferin was combined with $(Z\text{-}DEVD)_2$-rhodamine-110 or Ac-DEVD-AMC in the presence of caspase-3 with either a caspase-3 inhibitor (Ac-DEVD-CHO, 10 μM) or with a luciferase inhibitor (Resveratol, 5 μM). The luminescent signal from caspase-3 cleavage of Z-DEVD-aminoluciferin was read at 30 minutes, while the fluorescent signals from caspase-3 cleavage activity were read at 2 hours using the appropriate AMC or rhodamine 110 filter sets.

Results

Luminescence gave the largest signal to background ratio, followed by rhodamine-110, then AMC (Figure 6). All three substrates for detecting caspase-3 were consistently and negatively impacted by the addition of a known caspase-3 inhibitor. This suggests that luminogenic and fluorogenic reagents can be combined, e.g., to control for potential false interferences when either assay is performed. Thus, multiplexed signals can be used to determine if an agent is a true inhibitor of a particular enzyme.

Example III

Additional Exemplary Multiplex Assays

A. Multiplex assay for lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) in a single well format

The following detection reagents were prepared: 1) LDH reagent (30 mM HEPES, pH 7.4, 10 mM NaCl; 20 mM $MgSO_4$, 250 μM resazurin (Aldrich)) was used to reconstitute the lyophilized substrate component from CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Corp, Technical Bulletin 306); 2) ATP reagent (30 mM HEPES pH 7.4, 10 mM NaCl, 20 mM $MgSO_4$) was used to reconstitute the lyophilized substrate from CellTiter-Glo™ Luminescent Cell

Viability Assay (Promega Corp, Technical Bulletin 288); 3) LDH/ATP combination reagent (30 mM HEPES pH 7.4, 10 mM NaCl, 20 mM $MgSO_4$, 250 μM resazurin (Aldrich)) was used to reconstitute the lyophilized substrate component from CytoTox-ONE™, which in turn was used to reconstitute the lyophilized substrate from CellTiter-Glo™.

Sample dilutions of LDH (0, 1:8000, 1:4000, 1:2000, diamonds), ATP (0, 1.25, 2.5, and 5 μM, squares), and a combination of LDH/ATP (0/0 μM, 1:8000/1.25 μM, 1:4000/2.5 μM, and 1:2000/5 μM, respectively, triangles) were made with a 10 mM HEPES pH 7.5, 0.1% Prionex (PentaPharma Corp) solution, and 100 μl of the dilutions (n = 4) were added to wells of a white, 96-well plate. The appropriate detection reagent (100 μl) was added to the samples, the plates were protected from light, mixed for 30 seconds, and incubated at room temperature. Following an eight minute incubation, fluorescence was measured on a Labsystems Fluoroskan Ascent plate reader with filter set $560_{Ex}/590_{Em}$. At 30 minutes post-incubation luminescence was recorded using a Dynex MLX plate luminometer.

Results

There was a minor effect of LDH and its fluorogenic detection reagent on the luminogenic assay for ATP (Figure 7A) when compared to the control reaction (Figure 7C); however, detection of ATP was still possible. The addition of a luminogenic detection reagent to the fluorogenic assay for LDH did not affect background fluorescence (Figure 7B), and although overall fluorescence decreased when compared to the control reaction (Figure 7D) LDH activity was still detectable.

B. Multiplex assay for LDH and caspase-3 in a single well format

The following detection reagents were prepared: 1) LDH reagent- Caspase-Glo™ 3/7 Buffer supplemented with 238 μM resazurin was used to reconstitute the CytoTox-ONE™ lyophilized substrate; 2) caspase-3 reagent-the Caspase-Glo™ 3/7 buffer was used to reconstitute the Caspase-Glo™ 3/7 lyophilized substrate as per Promega Technical Bulletin 323; 3) LDH/caspase-3 combined reagent-LDH reagent (prepared as above) was used to reconstitute the lyophilized Caspase-Glo™ 3/7 substrate. The LDH detection reagent compound of the LDH/caspase-3 combined

reagent is unstable due to the presence of DTT in the Caspase-Glo™ 3/7 lyophilized substrate, so this reagent was prepared immediately prior to addition to samples.

Sample dilutions were prepared in 10 mM HEPES pH 7.5, 0.1% Prionex (PentaPharma Corp) solution: 0, 1:8000, 1:4000, 1:2000 dilutions of LDH (diamonds); 0, 5, 10, and 20 U/ml caspase-3 (BIOMOL Laboratories, squares), and a combination of LDH/caspase-3 (0/0 U/ml, 1:8000/5 U/ml, 1:4000/10 U/ml, and 1:2000/20 U/ml, respectively, triangles). 100 μl of the dilutions (n = 4) were added to white, 96-well plates. The appropriate detection reagent (100 μl) was added to the samples, and the plates were protected from light, mixed for 30 seconds, and incubated at room temperature. Following a six minute incubation at room temperature, fluorescence was measured on a Labsystems Fluoroskan Ascent plate reader with filter set $560_{EX}/590_{Em}$. At 45 minutes post-incubation luminescence was recorded using a Dynex MLX plate luminometer.

Results

There was a decrease in luminescence with the addition of a fluorogenic LDH detection reagent to a multiplex reaction when compared to a control reaction (Figures 8A and 8C, respectively). Despite the decrease in total luminescent signal in Figure 8A, the luminescent caspase-3 assay was functional in the presence of the fluorogenic LDH detection reagent. Figure 8B shows there was an increase in fluorescence background when the luminogenic caspase-3 detection reagent was added to the multiplex reaction when compared to control (Figure 8D); however, Figure 8A demonstrates the fluorogenic assay for LDH is functional in the presence of the luminogenic detection reagent for caspase-3. There was no contribution of LDH to background luminescence (Figure 8C), and there was no contribution of caspase-3 to background fluorescence (Figure 8D).

C. Multiplex assay for caspase-3 and protein kinase A (PKA) in a single well format

The following detection reagents were prepared: 1) PKA reagent-a 1X reaction buffer was prepared which contains 100 mM Tris pH 7.3, 100 mM $MgCl_2$, 1:1000 dilution of a PKA rhodamine-110 substrate (ProFluor™ PKA Assay, Promega Corporation, Technical Bulletin 315), and 400 μM ATP; 2) caspase-3 reagent-a 1X reaction buffer was prepared containing 100 mM Tris pH 7.3, 100 mM $MgCl_2$, 150 μg/ml recombinant thermostable luciferase, 80 μM Z-DEVD-aminoluciferin (Promega Corp), 400 μM ATP, 100 μM DTT (Promega Corp), 2.5 mM $CaCl_2$ (Fisher), 40 mM $MgSO_4$ (Fisher), and 0.2% Tergitol NP-9 (Sigma); 3) kinase/caspase-3 combined reagent-a 1X reaction buffer was prepared containing 100 mM Tris pH 7.3, 100 mM $MgCl_2$, 1:1000 dilution of a PKA rhodamine-110 substrate, 150 μg/ml recombinant thermostable luciferase, 80 μM Z-DEVD-aminoluciferin, 400 μM ATP, 100 μM DTT, 2.5 mM $CaCl_2$, 40 mM $MgSO_4$, and 0.2% Tergitol NP-9; 4) protein kinase stop reagent- a 1X stop reagent was prepared containing 100 mM Tris pH 7.3, 100 mM $MgCl_2$, 1:50 dilution of protease reagent (ProFluor™ PKA Assay), 30 μM staurosporine (BIOMOL Laboratories).

Sample dilutions were prepared in 10 mM HEPES pH 7.5, 0.1% Prionex (PentaPharma Corp) solution; 0, 1, 2, and 4 U/ml PKA (diamonds), 0, 5, 10, and 20 U/ml caspase-3 (squares), and a combination of PKA and caspase-3 (0/0 U/ml, 1/5 U/ml, 2/10 U/ml, and 4/20 U/ml, respectively, triangles), and 40 μl of the dilutions (n = 4) were added to white, 96-well plates. The appropriate detection reagent (40 μl) was added to the samples, the plates were protected from light, mixed for 30 seconds, and incubated at room temperature for 20 minutes. Following incubation, 40 μl of a protein kinase stop reagent were added to the wells which contained either the kinase reagent alone or the combination kinase/caspase-3 reagent. The plates were mixed an additional 30 seconds, protected from light, and incubated for 30 minutes longer at room temperature. Fluorescence was measured on a Labsystems Fluoroskan Ascent plate reader with filter set $485_{EX}/527_{Em}$. Luminescence was recorded using a Dynex MLX plate luminometer.

Results

The addition of a fluorogenic PKA assay detection reagent caused the luminescent background to increase (Figure 9A) when compared to the control reaction where PKA was present but the complete PKA detection reagent was absent (Figure 9C). However, reaction luminescence resulting from caspase-3 activity increased proportionately and the conditions did not appear to affect the luminogenic caspase-3 reaction itself. Addition of the detection reagent for caspase-3 to the fluorogenic assay for PKA (Figure 9B) decreased overall fluorescence by more than 50% when compared to the fluorescent control reaction (Figure 9D) where caspase-3 was present but the complete caspase-3 detection reagent was absent. Caspase-3 and PKA activities were measureable over background using these multiplex conditions.

D. Multiplex assay for *Renilla* luciferase and caspase-3 in a single well format

The following detection reagents were prepared: 1) EnduRen™ (Promega Corp.), a cell permeant modified coelenterazine substrate for *Renilla* luciferase, was diluted to 600 μM into F-12 tissue culture medium supplemented with 10% fetal bovine serum and 500 μg/ml G-418 sulfate; 2) caspase-3 substrate: $(Z\text{-}DEVD)_2$-rhodamine-110 (Promega Corp.) was diluted to 250 μM into F-12 tissue culture medium supplemented with 10% fetal bovine serum and 500 μg/ml G-418 sulfate; 3) luciferase/caspase-3 combined substrates:EnduRen™ (600 μM) and $(Z\text{-}DEVD)_2$-rhodamine-110 (250 μM) were diluted in F-12 tissue culture medium supplemented with 10% fetal bovine serum and 500 μg/ml G-418 sulfate.

CHO-K1 cells (ATCC) which stably express *Renilla* luciferase (CHO-K1 hRL25) were maintained in 10% fetal bovine serum and 500 μg/ml G-418 sulfate and used for cell based experiments. Experimental conditions utilizing these cells included: 1) varying levels of luciferase activity due to addition of staurosporine, 2) varying levels of luciferase activity due to staurosporine addition with caspase-3 enzyme addition, and 3) luciferase activity with no staurosporine but with addition of caspase-3 enzyme.

CHO-K1 hRL25 cells were harvested and plated into a 96-well clear bottom, white walled tissue culture plate at a density of 20,000 cells/well, and incubated

overnight at 37°C in 5% CO_2. Staurosporine at a final concentration of 0, 0.5, 1, 2 μM (10 μl/well) was added to the appropriate wells to initiate cell death, thus altering luciferase activity. Cells were incubated for an additional 3.5 hours at 37°C in 5% CO_2. Various concentrations of caspase-3 (BIOMOL Laboratories) were added to the appropriate wells at 0, 5, 10, and 20 U/ml, in tissue culture medium (10 μl/well). Therefore, combined staurosporine/caspase-3 concentrations for data points were 0 μM/0 U/ml, 0.5 μM/5 U/ml, 1 μM/10 U/ml, and 2 μM/20 U/ml, respectively. Immediately after addition of the caspase-3 enzyme, 10 μl/well of either luciferase substrate, caspase-3 substrate, or luciferase/caspase-3 substrates were added to the appropriate wells. After addition of the detection reagents, the plates were mixed briefly and incubated at 37°C in 5% CO_2 for two hours. Fluorescence was measured on a Labsystems Fluoroskan Ascent plate reader with filter set $485_{EX}/527_{Em}$. Luminescence was recorded using a Dynex MLX plate luminometer.

Results

Activity of *Renilla* luciferase was used in these assays as an internal control for cell death. Therefore, as staurosporine concentration increases, luciferase activity should decrease. Figure 10A shows that the addition of the caspase-3 substrate did not negatively affect the luciferase reaction when compared to the control reaction (Figure 10C). Figure 10B shows that the addition of the luciferase substrate had no effect on background fluorescence, even though there was a slight increase in total fluorescence when compared to Figure 10D. The luminogenic assay for *Renilla* luciferase was fully functional in the presence of caspase-3 or the caspase-3 substrate, and the fluorogenic assay for caspase-3 was only slightly affected, but fully functional, in the presence of *Renilla* luciferase or the *Renilla* luciferase substrate.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.